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(57) Abstract

Vacuum infiltration is an effective way of infecting *Eucalyptus* explants with *Agrobacterium*. It results in a high frequency of expression as shown by GUS histochemical assays. The procedure is highly time efficient and less damaging to the plant tissue, as wounding of cotyledon and leaf explants was not needed. Stably transformed plants could be regenerated from vacuum infiltrated explants. GUS histochemical assay and Southern hybridisation analyses confirmed the transformation. It has also been determined that the application of vacuum may be supplemented to the post application of pressure to create a transformationally effective pressure gradient.

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A METHOD OF TRANSFORMATION

This invention relates to a method of transformation.

This invention has particular but not exclusive application to a method of transforming explants with *Agrobacterium tumefaciens* and for illustrative purposes reference will be made to such application. However, it is to be understood that this invention could be used in other applications such as whole plant or seed transformation or with other infective vector systems.

Eucalyptus has been considered a difficult genus for transformation, with the main obstacle the low regeneration potential of explants. *Agrobacterium* mediated transformation is a commonly used method for transforming plants. To inoculate explants, the general practice is to incubate wounded explants with a suspension of *Agrobacterium*. Consequently, the transformation events occur mainly around the wound sites. In addition, there is the tedious, time-consuming, and painstaking work of wounding a large amount of explants. Further, wounding may cause tissue damage due to callus formation at the wound sites. Wounding causes a particularly high failure rate in regeneration of the explants.

New *in planta* transformation approaches have been developed, mainly in *Arabidopsis thaliana*. Seeds have been imbided with *Agrobacterium*, and plants allowed to grow to maturity in soil. Transformed plants were selected out from the harvested seeds. This method has had the advantage that no tissue culture is involved, but the transformation frequency is low.

Vacuum infiltration has been used as a means of agroinfection for introducing wheat dwarf virus into wheat embryos [Dale *et al.* Plant Sci 63: 237-245 (1989)]. This

technique of inoculation has also been used for introducing *Agrobacterium tumefaciens* into adult plants of *Arabidopsis*, where selected transformed seeds can be obtained after the infected plants reach maturity. [Bechtold *et al.*, C R Acad Soc Paris, Life Sci 316: 1194-1199 (1993), Bent *et al.*, Science 265: 1856-1860 (1994)]. More consistent results can be achieved using this new method. However, this method can probably be described as indirect transformation of germ cells and will have limited use when transformation targets are somatic cells. For example, in transformation of trees the targets are frequently the selected superior trees themselves, not the second generation from seeds, which are of different genotypes to the superior parent trees. Further, it may a substantial period of time for some plant species to reach maturity to enable selection of transformed seeds.

Recently, Norelli *et al.* [HortSci 31: 1026-1027 (1996)] tried to use vacuum infiltration as a way to inoculate explants with *Agrobacterium*. They found, however, that it did not enhance transformation frequency, nor change the distribution of the blue *GUS* pigmentation on apple leaf explants when compared with wounded explants infected in an *Agrobacterium* bath. Norelli et al. concluded that vacuum infiltration did not have a significant effect on transformation. Kapila *et al.*, [Plant Sci 122: 101-108 (1997)] has reported that up to 90% of the area of unwounded *Phaseolus vulgaris* leaves showed transient *GUS* expression following vacuum infiltration. However, there was no regeneration of the leaves after vacuum infiltration to obtain a transformed plant.

In each case, it is presumed that plant tissue cannot remain viable after being subjected to vacuum for extended periods of time. In the case of Kapila *et al.* above,

regeneration was not the point. It has now been surprisingly determined that stable transformation of certain plant tissue can be achieved with good transformation efficiency under conditions which would ordinarily be condemned as destructive of regeneration capacity. This has been established especially for *Eucalyptus* spp.

The present invention aims to alleviate at least one of the foregoing disadvantages and to provide a method of transformation which will be reliable and efficient in use. Other objects and advantages of this invention will hereinafter become apparent.

With the foregoing in view, this invention in one aspect resides broadly in a method of transformation of plant including the steps of:

immersing plant tissue in a medium including an infective transformation vector;

reducing the pressure on said tissue to -10 to -100 kPa gauge;

maintaining said pressure for 10 to 60 minutes, and raising said pressure to atmospheric pressure or above, said transformation vector being selected to provide integrating transformation of said plant tissue.

The aforementioned mentioned method has found particular application in the transformation of eucalypts. Accordingly in a further aspect, this invention in one aspect resides in a method of transformation of eucalypts including the steps of:

immersing eucalypt tissue in a medium including an infective transformation vector;

reducing the pressure on said tissue to -10 to -100 kPa gauge;

maintaining said pressure for 10 to 60 minutes, and raising said pressure to atmospheric pressure or above, said transformation vector being selected to provide

The eucalypt tissue may be selected from *E. grandis*, *E. tereticornis* or *E. camaldulensis*. The eucalypt tissue may be cell culture, a whole plant, an explant or germ material. In order to provide commercial material for prolongation of transformed plants it is preferred that the plant material be explants suitable for prolongation after transformation. The explants may be selected from shoots, cotyledons, hypocotyls, leaves, seedlings, or the meristem.

The infective transformation vector may be selected from any known suitable infective system to mediate transformation, such as a virus or bacterium. Preferably, the vector is *Agrobacterium*. *Agrobacterium* is the most common type of bacterium used for bacterially mediated-transformation of plants. However, it is envisaged that any other infective microorganism which may be proved to be effective in transformation of plant cells may be used. It is to be appreciated that with the rapidly growing development in biotechnology that other appropriate bacterium or other infective agent may be developed which might also be applicable.

The medium may be any liquid medium used to support the bacterial suspension without affecting the explant tissue. The medium may include chemicals which assist in transformation and regeneration with less tissue damage. For example, acetosyringone may be added to the medium. The transformation culture of *Agrobacterium* is preferably established at a population in the range 1-5 x 10^8 cfu ml^{-1}.

The reduced pressure may be achieved in any vacuum chamber or desiccator. It may be advantageous to use a vacuum vessel which is capable of maintaining a pressure above atmospheric as is described hereinafter. The vacuum vessel may be adapted to cycle the contents of the vessel through a range of pressures. It was found

It was found that prolonged exposure of plant tissue leads to hyperhydricity in the explant. The duration of the exposure is in the range of 10 to 60 minutes, the longer periods being preferred for recalcitrant species of eucalypt. Preferably, the duration of vacuum treatment is in the range of 15 to 20 minutes.

The pressure may be raised to atmospheric pressure or above slowly or stepwise. Preferably, the pressure is raised to atmospheric pressure or above very rapidly. This may be achieved by opening the vacuum chamber while still under negative pressure. Not being bound by theory, it is surmised that the vacuum (negative pressure) causes the air spaces between the cells in the plant tissue to decrease. The longer the duration and the lower the pressure of the vacuum the less air space within the plant tissue. The increase in pressure allows the infiltration medium including the infective transformation vector to relocate into the plant tissue. This is supported by the evidence of hyperhydricity from prolonged exposure in the vacuum.

Selection and regeneration of transformed explants may be performed by any method commonly used in the field tissue culture. For example, regeneration may include any of the steps of co-cultivation, incubation, callus induction, shoot induction and subculturing. Selection mediums or other selection methods typically used may be used for selecting successful transformants.

In a further embodiment, the multiple application of vacuum infiltration may also lead to improved effects in transformation and regeneration. For example, the reduced pressure effect may be provided by cyclically applying vacuum to the tissue rather than applying a vacuum for an extended period.

hitherto been unsuited to vacuum infiltration. It has been unexpectedly determined that the application of vacuum may be supplemented by the post application of pressure to create a transformationly effective pressure gradient.

In a further aspect, this invention resides in a method of transforming plants including the steps of:

immersing plant tissue in a medium including an infective transformation vector;

reducing the pressure on said tissue;

increasing said pressure to a pressure of at least 10 kPa above said reduced pressure to effect infiltration, and

regenerating said tissue.

The plant tissue may be cell culture, a whole plant, an explant or germ material. In order to provide commercial material for propagation of transformed plants it is preferred that the plant material be explants suitable for propagation after transformation. The explant may be selected from shoots, cotyledons, hypocotyls, leaves, seedlings, or the meristem.

The medium may include any infiltration media or bacterial suspensions typically used in vacuum infiltration procedures, or as described above.

The pressure differential between the reduced pressure and the over-pressure may in a suitable range so as not to cause damage or excessive hyperhydricity to the plant tissue. Particularly, the reduced pressure and time of maintenance at the reduced pressure is preferably selected to avoid hyperhydricity of the tissue. The corresponding over pressure is preferably selected to provide sufficient pressure differential between the reduced pressure and the over pressure to promote infiltration.

pressure may be in the range of 10 to 500 kPa. The plant material may be subjected to alternating cycles of said reduced and over pressures. For hardy species the pressure differential is preferably about 90 kPa or higher.

When the plant material is to be subjected to alternating positive and negative pressure, this may be achieved in any suitable vessel including a vacuum/pressure chamber or desiccator modified to allow the apparatus to function as a pressure vessel. Alternatively, the negative and positive pressure may be built by manual pulling and pushing of a piston of a syringe containing the plant material and medium. The piston may be pulled to a scale to create negative pressure or a vacuum. In opposition, the pushing of the piston creates a positive pressure. The pulling and pushing may performed repeatedly.

The inoculated plant material may be co-cultivated and normal selection and regeneration procedures used to obtain a transgenic plant.

In a further aspect, this invention resides in a method of transforming plants including the steps of:

immersing plant tissue in a medium including an infective transformation vector;

increasing said pressure to a pressure of at least 10 kPa to 500 kPa above the starting pressure to effect infiltration, and

regenerating said tissue.

In order that this invention may be more readily understood and put into practical effect, reference will now be made to the accompanying drawings which illustrate a preferred embodiment of the invention and wherein:

FIG. 1 is the control (*E. grandis*) seedlings, where the wounded explant

FIG. 2 is the high frequency *GUS* expression in vacuum-infiltrated *E. camaldulensis* seedling as assayed 5-6 days after inoculation;

FIG. 3 is the high frequency *GUS* expression in vacuum-infiltrated *E. grandis* seedlings as assayed 5-6 days after inoculation;

FIG. 4 is the high frequency *GUS* expression in vacuum-infiltrated *E. tereticornis* shoot as assayed 5-6 days after inoculation;

FIG. 5 is the regeneration of multiple *GUS*-positive shoots from *E. camaldulensis* callus obtained from vacuum-infiltrated explants;

FIG. 6 is a *GUS*-positive *E. grandis* shoot obtained from vacuum-infiltrated explants;

FIG. 7 is a *GUS*-positive *E. camaldulensis* plantlet obtained from vacuum-infiltrated explants;

FIG. 8 is a transgenic *E. grandis* plant in soil obtained from vacuum-infiltrated explants, and

FIG. 9 is a southern blot analysis of transformed *E. grandis* lines EG66 and EG77 that were obtained from vacuum infiltrated explants. The control was from non-transformed E. grandis. 35S*GUS*INT was used as the probe for hybridization. (a) undigested DNA samples. The absence of low molecular weight fragments indicated that there was no contamination of plasmid DNA. (b) DNA samples digested with *Hind*III.

EXAMPLE 1

Medium

The media used in this study were G22 and KG. G22 medium was used in a modified

were omitted. The phytohormone regimes in inductions medium were also different in using 1μM BA, 0:03-.01 μM TDZ (Sigma) and 1 μM NAA for callus induction of *E. grandis* and *E. tereticornis* and 1 μM BA and 0.5 μM NAA for *E. carnaldulensis*. The differentiation medium was a G22 medium supplemented with 2-5 μM BA and 0.5 μM NAA. A KG medium contain 0.2μM BA was used as the subculture medium for clone materials. All media was solidified with 0.25% Gelrite (Kelco, San Diego), and the pH was adjusted to 5.7 - 5.8 using KOH before autoclaving for 15 minutes at 121°C. Liquid medium, used for selecting transformed shoots, was KG supplemented with 0.01 μM BA and antibiotics.

Plant Materials

Seedlings of the two species (*E. grandis* and *E. camaldulensis*) used in this study were obtained by germinating sterile seeds on hormone-free KG medium. Cultures were maintained at 23°C under dim light (5-10 μMol m^{-2}). Seedlings aged 8-15 days were used as starting material for regeneration and transformation experiments. *E. grandis* and *E. tereticornis* clones maintained on KG medium were also used as starting material. Wounding of explants, when necessary was performed using the tip of a scalpel blade or a sharpened needle.

The general protocol is as follows:

a. Clones are sub-cultured every 3-5 weeks on KG media [Laine E and David A: Plant Cell Rep. 13: 473-476 (1994) supplemented with 2 μM BA (6-benzlaminopurine).

b. At subculture, a piece of shoot consisting of one or two nodes are cut off from the young shoots and used for the initiation of new cultures. If the leaves are too big,

c. Eight to ten node cultures are kept in a 250 ml jar (bunzel) containing 40 ml of medium.

d. The jars are kept at 23°C under dim light (5-10 μMol m^{-2}) with a 12-hour photo-period. Under such growth conditions, the shoot cultures are generally clusters consisting of 3 - 10 short shoots (5-20mm) with small leaves (~ 5mm).

e. For transformation, single shoots were excised and the leaves at the base are removed. The young leaves, generally the top 2 - 3 pairs are retained for transformation.

This particular protocol was optimised for use with *Eucalyptus*, but it is not restricted to said genus and is generally envisioned to be applicable to other plant genus/species, particularly those which lend themselves to clonal micro-propagation. One aspect of this method is designed to clonally propagate large numbers of suitable plant starting material for use in transformation. In doing so, this methodology is genotype independent, as it is capable of generating as much starting material as desired from a given genotype. Even very low regeneration frequencies can be accomodated. From this material, different plant tissues can be isolated for transformation (e.g. stem or leaves). Transformation into the clonal plant starting material may be by several transformation methods, including, but not limited to, vacuum infiltration.

Agrobacterium and vacuum infiltration

INT. This vector carries a *GUS* gene interrupted by a plant intron. Consequently, *GUS* expression is exclusively from plant cells, and contamination from stained bacteria are excluded.

Agrobacterium were grown overnight in a liquid YEP medium at 28°C until the OD_{600} value was around 1. The bacterium suspension was diluted to the desired density of 1-5x10^8 ml^{-1} (6-30 times) using a liquid KG medium. Acetosyringone was added at a final concentration of 50 µM. For vacuum infiltration, the plant materials were placed in an *Agrobacterium* suspension. The mixture was put into a desiccator, and vacuum infiltrated -95 kPa for 20 minutes in a BioHood. As the control, explants were immersed in an *Agrobacterium* bath for 20 minutes. After inoculation, the explants were blotted dry with sterile filter paper before transfer to an MG22 co-cultivation medium. The cultures were kept on the co-cultivation medium, in the dark for 2-5 days, depending on the degree of *Agrobacterium* overgrowth.

Selection

Following 2-5 days incubation on the co-cultivation medium, cotyledon and leaf explants were placed on a solid callus induction medium containing 250 mg l^{-1} cefotaxime or 100 mg l^{-1} Timentin (SmithKline Beecham), and 0-30 mg l^{-1} geneticin (Sigma). The cultures were subcultured onto fresh medium every 2-3 Weeks. When shoots emerged, they were transferred to a liquid medium containing 0.01µM BA and antibiotics (50 mg l^{-1} cefotaxime or 100 mg l^{-1} Timentin) and 2.5 or 5 mg l^{-1} geneticin for further selection. Shoots which survived the liquid selection were subcultured in fresh liquid medium containing 5 mg l^{-1} geneticin. During the first 3 weeks of callus induction

under light (5-10 μMol m^{-2}).

Assays for Transformation

For *GUS* histochemical assays explants were incubated in an X-gluc solution (0.5mg l^{-1} X-gluc in Phosphate buffer, pH 7.0) overnight at 37°C. NPTII enzyme assay was performed. For Southern blot analysis, DNA was extracted from frozen eucalypt leaves using Genomic-tip 100G Kit (QIAGEN). Purified DNA (20 μg) was digested with *Hind*III, separated by electrophoresis on 0.7% agarose gel, blotted onto Hybond N+ membrane (Amersham) and hybridised using a labeled, random-primed internal *Hind*III fragment of 35S*GUS*INT containing the *GUS* gene as a probe.

Transient Expressions

Factors affecting transformation efficiency, including density of *Agrobacterium*, vacuum pressure and duration of vacuum treatment, were studied. Two pressure settings were tested. Transient expression was high when vacuumed at -95 kPa rather than at -45 kPa. Using *Agrobacterium* at a density of 1×10^8 or 5×10^8 ml^{-1} had no obvious effects on the level of transient expression. Time of vacuum treatment in the tested range (10 to 60 minutes at -95 kPa) appeared to have very little effect on the level of transient expression of *E. grandis*. However, prolonged exposure at the lower pressure resulted in more severe hyperhydricity of explants following the vacuum treatment. Therefore, the time for vacuum treatment was chosen as 20 minutes for *E. grandis*, and 15 minutes for *E. camaldulensis*.

Distribution of blue cells on vacuum infiltrated *Eucalyptus* explants was notably

wounded explants in an *Agrobacterium* bath for 20 minutes. On wound explants inoculated an *Agrobacterium* bath, blue areas or single blue spots were usually observed around the wound sites. Sometimes, single blue spots were seen away from the wound sites, but generally still within a close distance, as seen in Fig. 1.

In contrast, there were many single blue spots or blue areas distributed on the whole vacuum infiltrated leaf or cotyledon explant, in addition to the blue areas and cells around the wound sites, as seen in Figs. 2 - 4. These randomly distributed blue spots and areas were also observed at similar frequencies on vacuum infiltrated cotyledons and leaves that had not been wounded. In addition to the individual blue spots, large continuous blue areas were frequently observed, which could cover 50-90% of the area of the cotyledon or leaf, as also seen in Figs 2 - 4. Occasionally, when whole shoots of *E. grandis* and *E. tereticornis* were vacuum infiltrated, the whole meristem area was stained blue by GUS pigmentation. For hypocotyls and internodes, the pattern of *GUS* expression of vacuum infiltrated explants was similar to those inoculated in an *Agrobacterium* bath, i.e. the expression was predominantly at the wound sites (the cut ends).

Regeneration of Transformed Plants

On induction medium, vacuum infiltrated leaves and cotyledons were darker green than control explants. They were also slightly swollen, with an appearance similar to vitrificated (hyperhydric) cultures. However, regeneration potential of the explants appeared not to be affected. In one experiment, explants from *E. grandis* seedlings were vacuum infiltrated under -95 kPa for 20 minutes before being placed

and 14 of the 30 hypocotyls formed shoots, a similar regeneration frequency (50%) to the average regeneration experiments.

Transgenic plants, referring to Figs. 5-8, were obtained from explants vacuum infiltrated for 20 minutes under -95 kPa in a liquid containing 1-5x10^8 ml^{-1} *Agrobacterium*. The explants were co-cultivated in MG22 medium for 2-3 days before subculturing onto medium containing 250 mg l^{-1} cefotaxime or 100 mg l^{-1} Timentin for 4-5 days. They were then transferred onto medium containing 10 mg l^{-1} geneticin for 10 days, following by 15mg l^{-1} geneticin for 2 weeks, and finally kept on 30 mg l^{-1} geneticin until plant formation.

Four putatively transformed (*GUS* positive) plants of *E. grandis* were regenerated from 278 cotyledon (1.4%), 3 putatively transformed plants of *E. camaldulensis* were regenerated form 150 cotyledons (2.2%), and a one putatively transformed plant of *E. camaldulensis* was regenerated from 100 hypocotyls (1.0%). These frequencies were similar to those for wounding followed by inoculation in an *Agrobacterium* bath.

Transformation of plants was confirmed by positive *GUS*-histochemical staining (Figs. 1 - 8), NPTII enzyme assay (not shown), and Southern blot hybridisation analysis (Fig. 9). Southern hybridization analysis was performed to confirm the presence of the 35S*GUS*INT transgene into the plant genome in two putatively transformed E. grandis lines EG66 and EG67, and a non-transformed control. Hybridisation, with ^{32}P-labeled *GUS* gene from 35S*GUS*INT vector as a probe, shows strong signals corresponding to high molecular weight DNA (> 23kb) in lines EG66, EG67. The DNA extracted from the control, non-transformed plant, did not hybridise

the genomic DNA bands. These results indicate the integration of the 35S*GUS*INT plasmid into the *Eucalyptus* genomic DNA, and that no contamination with plasmid DNA had occurred.

The DNA samples were digested with *Hind*III, which released a 2.8 kb internal fragment of the T-DNA, containing the *GUS* gene sequence with 35S promoter and terminator sequences. A comparison of the intensities of the bands indicates that EG66 and EG67 have multiple (three to four) copies of 35S*GUS*INT transgene, as seen in Fig. 9. No detectable signal was observed in the control.

Eucalyptus has been considered a difficult genus for transformation, with the main obstacle the low regeneration potential of explants. Methods in accordance with the foregoing embodiment results in an increase in regeneration capacity by vacuum infiltration, due to the minimisation of tissue damage by avoidance of wounding. It is to be appreciated that better results than those obtained in *Eucalyptus* could be expected if this method is applied to other plant species whose leaf and cotyledon explants have a greater potential for regeneration. It is to be appreciated that factors such as the density of the bacterium suspension, the vacuum pressure, and the duration of the vacuum treatment will vary in relation to the type of plant material used and the type of plant species.

One feature of vacuum infiltration inoculation is the occurrence of infection is not limited to the wound sites, but is widely spread across the whole leaf blade. Transient expression on cotyledons and leaves was frequently so high that it was impossible to count the blue spots to quantitatively present transformation results. This high level of transformation is advantageous in plants where whole leaf or cotyledon explants

from a larger tissue area.

EXAMPLE 2

Negative/Positive Pressure Inoculation

Two experiments were conducted trying to use a syringe to produce positive/negative pressure to introduce *Agrobacterium* into eucalypt plants.

10 day old seedlings of *E. camaldulensis* were used. After cutting off the root ends, the explants (the intact cotyledons attached to the hypocotyls) were put into a 50 ml plastic syringe to which 20 ml of *Agrobacterium* (AGL1) suspension was added. The negative and positive pressure was built by manual pulling and pushing of the piston. The piston was pulled to the scale of 50 ml to create the negative pressure (vacuum). The pushing to give positive pressure was difficult to perform and impossible to measure. Pulling and pushing were performed repeatedly for 5 minutes and the explants were allowed to stay in the syringe for an extra 10 minutes before blotting dry using a filter paper and transferred to co-cultivation medium. Seedlings inoculated in an *Agrobacterium* bath for 30 minutes were used as the experimental control. The inoculated seedlings were allowed to stand on a KG medium 0.2 µM BA) for 3-5 days before the cotyledon and hypocotyls were excised. The excised cotyledons and hypocotyls were then transferred to a selection/regeneration medium and subcultured as in routine transformation experiments.

Transient Expression

Transient expression of the *GUS* gene was assayed in the first experiment. 15 of 18

cells. Only 2 out of 20 cotyledons (10%) and 6 out of 11 (55%) from the control had blue cells.

Stable Transformation

GUS expression in calluses was assayed in the second experiment using cultures maintained on selection medium for 3 weeks. Two of six assayed cotyledon calluses and two of three hypocotyl calluses were found to be *GUS*-positive. Four hypocotyl calluses from the control were all negative.

Transformed Shoot

One *GUS*-positive shoot was obtained following 6 weeks' incubation on selection medium. The shoot was obtained from the first experiment, where 30 seedlings were used as starting material. Ten seedlings were assayed for transient expression and the shoot was obtained from one of the remaining 20 hypocotyls.

In use, vacuum infiltration is an effective way of infecting *Eucalyptus* explants with *Agrobacterium*. It results in a high frequency of expression as shown by *GUS* histochemical assays. The procedure is highly time efficient and less damaging to the tissue, as wounding of cotyledon and leaf explants was not needed. Stably transformed plants could be regenerated from vacuum infiltrated explants. *GUS* histochemical assay and Southern hybridisation analyses confirmed the transformation.

It will of course be realised that while the foregoing has been given by way of

thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as is herein set forth.

CLAIMS

1. A method of transformation of plant tissue including the steps of:

immersing plant tissue in a medium including an infective transformation vector;

reducing the pressure on said tissue to -10 to -100 kPa gauge;

maintaining said pressure for 10 to 60 minutes, and

raising said pressure to atmospheric pressure or above, said transformation vector being selected to provide integrating transformation of said plant tissue.

2. A method of transformation according to Claim 1, wherein said plant tissue comprises Eucalyptus explants.

3. A method of transformation of eucalypts including the steps of:

immersing eucalypt tissue in a medium including an infective transformation vector;

reducing the pressure on said tissue to -10 to -100 kPa gauge;

maintaining said pressure for 10 to 60 minutes, and

raising said pressure to atmospheric pressure or above, said transformation vector being selected to provide integrating transformation of said eucalypt tissue.

4. A transformation method according to Claim 3, wherein eucalypt tissue is selected from *E. grandis, E. tereticornis* or *E. camaldulensis.*

5. A transformation method according to any one of claims 3 or 4, wherein the

material.

6. A transformation method according to Claim 5, wherein the tissue is explants selected from shoots, cotyledons, hypocotyls, leaves, seedlings, or meristem.

7. A transformation method according to any one of the preceding claims, wherein the infective transformation vector is selected from a bacterial or viral infective system.

8. A transformation method according to Claim 7, wherein the infective transformation vector is *Agrobacterium*.

9. A transformation method according to Claim 8, wherein the medium includes chemicals which assist in transformation and regeneration with less tissue damage.

10. A transformation method according to Claim 9, wherein acetosyringone is added to the medium.

11. A transformation method according to any one of claims 8 to 10, wherein the transformation culture of *Agrobacterium* is established at a population in the range 1-5 x 10^8 cfu ml^{-1}.

12. A transformation method according to any one of the preceding claims, wherein said reduced pressure is achieved in a vacuum chamber or desiccator.

13. A transformation method according to Claim 12, wherein said vacuum chamber comprises a vacuum vessel which is capable of maintaining a pressure above atmospheric.

14. A transformation method according to Claim 13, wherein said vacuum vessel is adapted to cycle the contents of the vessel through a range of pressures.

15. A transformation method according to Claim 3, wherein said reduced pressure is -95 kPa gauge.

16. A transformation method according to Claim 4, wherein the duration of vacuum treatment is in the range of 15 to 20 minutes.

17. A transformation method according to Claim 13, wherein the pressure is rapidly raised to atmospheric pressure or above.

18. A transformation method according to any one of the preceding claims, wherein said reduced pressure comprises cyclically applying vacuum to said tissue.

19. A method of transforming plants including the steps of:

immersing plant tissue in a medium including an infective transformation vector;

reducing the pressure on said tissue;

increasing said pressure to a pressure of at least 10 kPa above said reduced

regenerating said tissue.

20. A transformation method according to Claim 19, wherein said reduced pressure and a time of maintenance at said reduced pressure is selected to avoid hyperhydricity of said tissue.

21. A transformation method according to Claim 20, wherein said over pressure is selected to provide sufficient pressure differential between said reduced pressure and said over pressure to promote infiltration.

22. A transformation method according to Claim 21, wherein said pressure gradient between the reduced pressure and the over-pressure is in the range of 10 to 500 kPa.

23. A transformation method according to any one of claims 19 to 22, wherein said plant material is subjected to alternating cycles of said reduced and over pressures.

24. A method of transforming plants including the steps of:

immersing plant tissue in a medium including an infective transformation vector;

increasing said pressure to a pressure of at least 10 kPa to 500 kPa above the starting pressure to effect infiltration, and

regenerating said tissue.

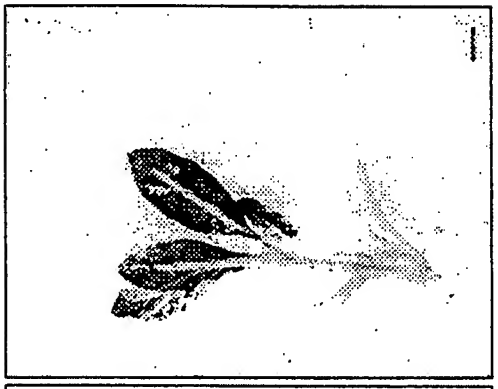


Fig. 4

Fig.3

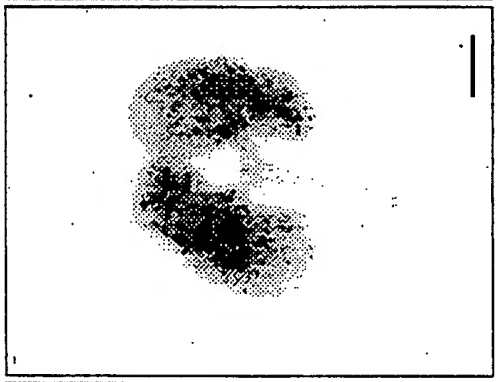


Fig. 2

Fig. 1

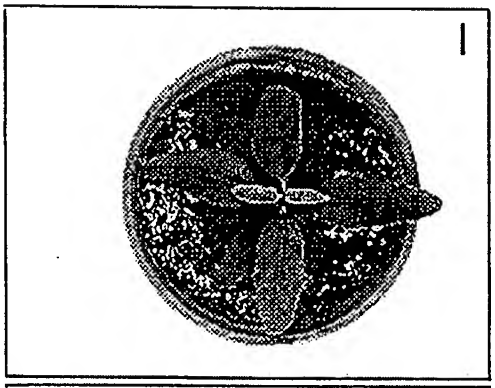


Fig. 8

Fig. 7

Fig. 6

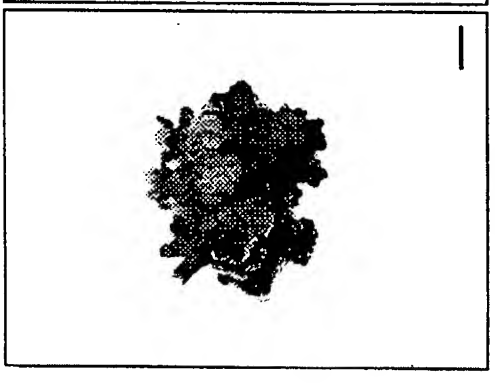
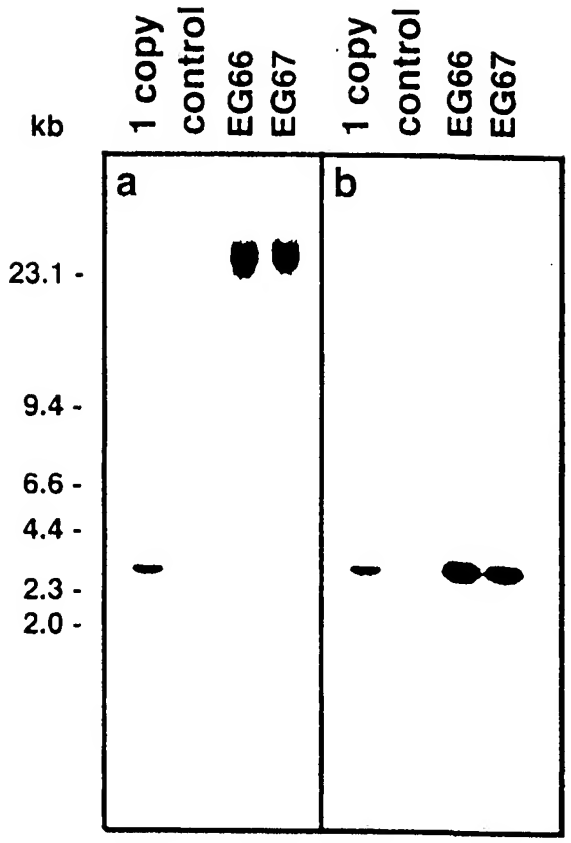


Fig. 5


kb
1 copy control
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b
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9.4 -
6.6 -
4.4 -
2.3 -
2.0 -

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00195

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A01H 4/00, C12N 15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Databases below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: A01H/IC, C12N-015/IC, C12N-005/IC, vacuum() infiltration#, reduced() pressure#, negative() pressure#
CHEMICAL ABSTACTS: tissue culture/ct, plant tissue culture/ct, vacuum infiltration, reduced pressure, negative pressure
MEDLINE: tissue culture/ct, vacuum infiltration, reduced pressure, negative pressure

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU 18075/88 (CROP GENETICS INTERNATIONAL) 1 December 1988 See entire document, in particular examples 4, 9, 11, 15.	1-12,15,16
X	C.R. Acad. Sci. Paris (1993) volume 316, pages 1194-1199 by Bechtold N et al. "In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants" See entire document, in particular page 1195, column 2	1-12,15,16

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
18 May 1998

Date of mailing of the international search report
27 MAY 1998

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00195

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Plant Science (1989) volume 63, pages 237-245 by Dale PJ et al. "Agroinfection of wheat innoculation of in vitro grown seedlings and embryos" See entire document.	1-12,15,16

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00195

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	18075/88	CA	1320352	EP	358718	IL	86466
		WO	8809114	US	5415672	ZA	8803556
							END OF ANNEX